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Functional study of eIF5A in *Nicotiana tabacum* by Riobosome-seq

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Abstract

Tobacco serves as a crucial economic crop globally, having significantly propelled the development of human society. In this study, *Nicotiana tabacum* was employed as the research model to explore the potential biological functions of 10 genes of the eIF5A family, which contributes to an in-depth understanding of the potential gene redundancy phenomenon. As a eukaryotic translation initiation factor, eIF5A exhibits high conservation across plant species, including *N. tabacum*. A total of 1,019 differentially expressed genes (DEGs) were identified in, among which 656 were upregulated and 363 downregulated specifically at the translational level, rather than the transcriptional level. The *elf5a^{mut}* exhibits extremely obvious phenotypic characteristics and is shorter than the wild type(K326). Through Gene Ontology (GO) and KEGG enrichment analyses of these DEGs, they were found to be significantly enriched in photosynthesis-related pathways, including roles in light capture and the composition of the photosynthetic system. This finding is essential for understanding the roles of the eIF5A gene and its hypusination modification in plant development and stress responses. Additionally, prediction of the protein-protein interaction (PPI) network of eIF5A revealed potential interactions with proteins such as DHS1, suggesting its involvement in Deoxyhypusine synthesis via PPI. This study

systematically explores the biological functions of eIF5A in *N. tabacum*, filling a research gap in the current understanding of eIF5A in tobacco. The findings lay a foundation for further investigations aimed at improving tobacco quality.

Key Words: *Nicotiana tabacum*, eIF5A, Riobosome-seq, translation

Statements and Declarations

The authors declare that there is no conflict of interest.

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Key Message

The eIF5A gene affects the translation level of *Nicotiana tabacum* but not the transcription level;

It may regulate the life processes of *Nicotiana tabacum* by influencing its photosynthesis.

It plays an important role in regulating the life activities of *Nicotiana tabacum* through potential interactions with many proteins, such as DHS.

Introduction

Nicotiana tabacum, commonly known as cultivated tobacco, is an allotetraploid (2n=4x=48) model plant in the field of biological research, renowned for its economic significance and biological complexity(Ahmed et al. 2020; Song et al. 2025).The genes of *N. tabacum* can be edited or exogenous genes can be introduced into it, commonly used for plant gene function verification, plant senescence(Gao et al. 2020; Li et al. 2019; Xu et al. 2025), cell wall polysaccharide metabolism(Obembe et al. 2007; Qu et

al. 2024), axillary bud development(Wang et al. 2022), plant photoperiod(Puzanskiy et al. 2024), plant nutrition(Fatica et al. 2019), photosynthesis (Raza et al. 2021), and other directions.

Eukaryotic translation initiation factor 5A (eIF5A) as a particular protein found in all eukaryotic kingdoms, which is the only protein known to contain a unique amino acid modification, hypusine(Hopkins et al. 2008; Chamot et al. 1992), playing an important role in the growth and development of plants(Feng et al. 2007; Belda-Palazon et al. 2016)and in coping with adverse stress(Palfi et al. 2021; Hopkins et al. 2008). Most studies on eIF5A have been reported in humans and yeast, with only a few studies involving plants. In yeast, there are two genes encoding eIF-5A (TIF51A and TIF51B), with a homology of 90%. The inactivation of TIF51A expression leads to slow growth of yeast cells, while the mutation of TIF51B causes yeast cells to stop growing. If both TIF51A and TIF51B are inactivated simultaneously, yeast cells cannot survive(Joachim et al.1991). Studies conducted by Marianne T. Hopkins(Hopkins et al. 2008)have indicated that eIF5A is involved in the development of disease symptoms induced by a common necrotrophic bacterial phytopathogen in Arabidopsis (*Arabidopsis thaliana*). Danuta Chamot(Chamot et al. 1992) cloned and identified the eIF5A homologs in tobacco and speculated that Neif-5A2 is a housekeeping protein and Neif-5A1 may regulate the light-dependent translation of specific transcripts. Wang(2012) expressed the TaeIF5A in poplar and transgenic yeast ,the homologous gene of eIF5A , enhancing the stress resistance of plants through mechanisms such as promoting protein synthesis and enhancing the ability to scavenge reactive oxygen species (ROS). In addition to its crucial role in protein translation, eIF5A has also been reported to be involved in cell cycle transition(Zanelli et al. 2005) and even associated with mRNA degradation(Dorit et al. 1998; Sandro et al. 2002; Xu et al. 2001; Aiguo et al. 2004).

There are ten homologous genes of the eIF5A family in *N. tabacum*, namely eIF5A-1, eIF5A-2, eIF5A-3, eIF5A-4, eIF5A-5, eIF5A-6, eIF5A-7, eIF5A-8, eIF5A-9, and eIF5A-10. At present, there are very few studies on the eIF5A in *N. tabacum*, and as a

result, little is known about its specific roles in biological processes. To comprehensively understand the functions, characteristics of eIF5A and its role in biological processes, it is urgent to conduct more in - depth explorations in various aspects such as the central dogma, genetic regulation, and stress responses.

By the time, the advancement of sequencing technology in protein translation levels greatly enriched the content of omics research in the biological field. The combination of mathematical modeling and omics measurements showed, translational regulation, one of the four major regulatory processes of the proteome in the central dogma, accounts for more than half of all regulatory processes in life and is the most important regulatory step(Zhao et al. 2019; Schwanhäusser et al. 2011). Translatome research plays a crucial role in fields such as revealing the regulatory mechanisms of gene expression(Li et al. 2023), biological evolution research(Zhulyn et al. 2023), and disease diagnosis(He et al. 2024). It has opened a new door for life science research.

To clarify the role of eIF5A in *N. tabacum*, we obtained the *eif5a^{mut}* and measured its translatome level. By comparing the changes in the translation level with those of the wild type (K326), we captured 1,019 differentially expressed genes and conducted further research on these differentially expressed genes, laying the foundation for the in-depth analysis of the eIF5A in the future.

1. Materials and methods

1.1 plants and its growth conditions

We used tobacco K326 as the wild type and *eif5a^{mut}* as the experimental materials, which knocked out the 10 family genes of eIF5A (Table 1, Supplementary Data Set S1) in the background of K326. The seeds of allotetraploid tobacco K326 were provided by the Technology Center, China Tobacco Hunan Industrial Co., Ltd. Plants were grown in a greenhouse with a photosynthetic photon flux density (PAR) of 80-250 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and a 16-h light/8-h dark photoperiod. The temperature was 25 ± 2 °C, and the relative humidity was $60 \pm 2\%$.

Table 1 The sequence characteristics of eIF5A genes in *Nicotiana tabacum*.

Gene name	Gene ID	Amino Acid(aa)	MW(kDa) ¹	PI ²	GRAVYB ³
eIF5A-1	Ntab0807350.1	159	17.4	5.71	-0.459
eIF5A-2	Ntab0888390.1	159	17.4	5.71	-0.457
eIF5A-3	Ntab0437010.1	159	17.4	5.29	-0.487
eIF5A-4	-	160	17.5	5.61	-0.544
eIF5A-5	Ntab0589980.1	160	17.5	5.78	-0.546
eIF5A-6	Ntab0516850.1	159	17.4	5.48	-0.447
eIF5A-7	Ntab0203540.1	160	17.4	5.61	-0.494
eIF5A-8	Ntab0450160.1	160	17.6	5.78	-0.549
eIF5A-9	Ntab0847410.1	159	17.4	5.60	-0.489
eIF5A-10	-	159	17.4	5.48	-0.447

¹ the Molecular weight of proteins (kDa); ² the isoelectric point; ³ the grand average of hydropathicity, a negative value indicates that the protein is generally hydrophilic

1.2 Ribosome Isolation

1.2.1 Seed germination and Seedling grinding

After sterilizing the seeds of the K326 and the *eif5a^{mut}*, placed them on 1/2 Murashige and Skoog (MS) medium and vernalize them at 4 degrees Celsius for about 7 days. Subsequently, transfer them to incubator with a temperature of 28 degrees Celsius and a humidity of 80% and culture them for approximately 7 days. When the roots of the seedlings reach about 1 centimeter in length and the two cotyledons are fully expanded, place the seedlings in liquid nitrogen and grind them, polysome extraction buffer (PEB, Table 2) needs to be added during the grinding process. Pay attention to removing impurities such as the culture medium as much as possible. After grinding well, add an appropriate amount of RNase inhibitor. Dispense the samples into sterile and enzyme-free 1.5 ml Eppendorf tubes.

Table 2 Preparation of Polysome Extraction Buffer (PEB).

Reagent	Working concentration	Volume(10ml)
Tris-acetate	40mM	500μl
Sucrose	0.2M	0.685g

KCl	0.2M	1ml
MgCl ₂	15mM	500μl
Chloramphenicol	100μg/ml	40μl
Cycloheximide	100μg/ml	40μl
TritonX-100	1%	100μl
Polyxyethylene (10) tridecyl ether	2%	200μl
β-Mercaptoethanol	20mM	10μl
RNA inhibitor	-	10μl
Protease inhibitor	-	A quarter tablet
DEPC water	-	Up to 10ml

1.2.2 Preparation of Sucrose Gradient Concentrations

This step is completed by a fully automatic density gradient preparation system. Use sucrose cushion buffer (SCB, Table 3) to prepare sucrose solutions with mass fractions of 10% and 50% respectively. Add the two concentrations of sucrose solutions to the instrument at a ratio of 1:1 and start the instrument to generate a 10%-50% sucrose gradient concentration solution. The instrument parameters can be adjusted according to own needs.

Table 3 Preparation of Sucrose Cushion Buffer (SCB).

Reagent	Working concentration	Volume(50ml)
Tris-acetate	40mM	2ml
KCl	0.1M	2.5ml
MgCl ₂	15mM	2.5ml
Chloramphenicol	100μg/ml	200μl
Cycloheximide	100μg/ml	200μl
DEPC water	-	Up to 50ml

1.2.3 Isolation of Target Ribosomes

Place the sample on the upper layer of the sucrose gradient concentration solution and centrifuge it in an ultra-high speed refrigerated centrifuge at 4°C, and 240000g for 3 hours. After centrifugation, collect the target ribosomes bound to RNA in the sample through the separation system, and set the system parameters according to own needs.

1.3 Analysis of the eIF5A

The CDS sequences of the eIF5A from other 19 plant species were downloaded from the NCBI database (Supplementary Data Set S2). Using MEGA(Kumar et al. 2018) software, all tested species were subjected to nucleotide sequence alignment. A phylogenetic tree was constructed by the Neighbor-Joining method, with Bootstrap test parameters set to 1,000 repetitions, and other parameters using system defaults.

Amino acid sequence alignment of the eIF5A in the tested species was performed using MEGA software, and the results were visualized in BioEdit 7.7 software.

1.4 Screening of different expressed genes

The DESeq2(Love et al. 2014) software is used to standardize the counts of genes in each sample (using the BaseMean value to estimate the expression level), calculate the fold - change, and conduct a significance test for differences using the NB (negative binomial distribution test). Finally, differentially expressed protein - coding genes are screened based on the fold-change and the results of the significance test for differences. For non - biologically replicated samples, DESeq(Anders et al. 2012) is used for differential analysis by default.

1.5 Gene Ontology (GO) enrichment analysis

After obtaining the DEGs, perform GO enrichment analysis on these genes and describe their functions (in combination with the GO annotation results). Method for GO functional enrichment analysis(Gene Ontology 2015; Chen et al. 2017): Count the number of differentially expressed genes included in each GO term, and use the

hypergeometric distribution algorithm to calculate the significance of the enrichment of differentially expressed genes in each GO term. The calculation results will return a P - value for the enrichment significance (calculate each term in BP, CC, and MF using Fisher's exact test respectively). A lower value indicates greater statistical significance. Genes for subsequent research can be selected based on the results of the GO analysis combined with biological significance.

$$p = 1 - \sum_{i=0}^{m-1} \frac{\binom{M}{i} \binom{N-M}{n-i}}{\binom{N}{n}}$$

$$Enrichment\ score = \frac{m}{n} / \frac{M}{N}$$

The formula for calculating the p-value using the hypergeometric distribution test and the formula for calculating the Enrichment score.

Among them, N represents the number of genes with GO annotations among all genes; n represents the number of genes with GO annotations among the differentially expressed genes within N; M represents the number of genes annotated to a specific GO Term among all genes; m represents the number of differentially expressed genes annotated to a specific GO Term.

1.6 Enrichment analysis of DEGs in the KEGG pathway

KEGG(Kanehisa et al. 2007) is the main public database related to Pathway. The KEGG database is used to conduct Pathway analysis on differentially expressed protein-coding genes (combined with the KEGG annotation results), and the hypergeometric distribution test method is employed to calculate the significance of the enrichment of differentially expressed genes in each Pathway entry.

1.7 Prediction of Protein-Protein interaction

The protein-protein interaction network of eIF5A is analyzed using STRING(Szklarczyk et al. 2023) with reference to the *N. tabacum* database. The results

are output in TSV format and imported into Cytoscape for visualization. Meanwhile, they are also output in SVG format and imported into Adobe Illustrator for beautification.

1.8 Quantitative PCR of *elf5a^{mut}* for Verification

A SuperScript IV First-Strand Synthesis System (Thermo Fisher, 18091050) was used for RT-qPCR. Note: (i) The template RNA should be heated at 95°C for 3 min to linearize its secondary structure; (ii) an RT reaction can be performed with at most 5 gene-specific primers; and (iii) for the RT reaction, increase the temperature to 65°C if using gene-specific primers. The primers are listed in Supplemental Table S3.

Detailed information on the manufacturers, versions and other relevant specifications of the reagents, instruments and software used in the aforementioned experimental protocol is provided in Table 4.

Table 4 Manufacturers and version numbers of softwares

Name	Version	Manufacture	URL
Green house	QHP-460BE	Mingtu Technology	-
Ultracentrifuge	OptimaTML-100XP	Beckman	-
Automatic density gradient preparation system	Biocomp	Beckman	-
MEGA	MEGA 10	Arizona State University	-
BioEdit	7.2.5	Tom Hall	-
DESeq	1.38.0	Bioconductor	-
DESeq2	1.24.0	Bioconductor	-
Adobe Illustrator	28.7.1	Adobe Systems	-

Photo Shop	25.12.4	Adobe Systems	-
GraphPad Prism	10.3.1	GraphPad Software	
STRING	-	-	https://string-db.org/
NCBI	-	-	https://www.ncbi.nlm.nih.gov/
Reagent	-	SIGMA	-

2.Results

2.1 Mutant Generation

We knocked out 10 homologous family genes of eIF5A using CRISPR technology in the background of K326 (Supplementary Data Set S1) and obtained *eif5a^{mut}* (the sequences of eIF5A-6 and eIF5A-10 were completely identical, so discussions on eIF5A-10 were omitted in subsequent experiments). For eIF5A-1, 107 lines were successfully knocked out, including 14 homozygous lines; eIF5A-2 had 86 knocked-out lines, 16 of which were homozygous; eIF5A-3 had 117 knocked-out lines, 20 of which were homozygous; eIF5A-4 had 111 knocked-out lines, 20 of which were homozygous; eIF5A-5 had 32 knocked-out lines with no homozygous lines, so we further knocked out eIF5A-5-2 and obtained one homozygous line; eIF5A-6 had 47 knocked-out lines, including 1 homozygous line; eIF5A-7 had 117 knocked-out lines, 13 of which were homozygous; eIF5A-8 had 32 knocked-out lines with no homozygous lines; eIF5A-9 had 124 knocked-out lines, including 11 homozygous lines. Most mutations in the *eif5a^{mut}* were single-base mutations or single-base deletions (Figure 1A and B). We then performed RT-qPCR experiments and verified that the expression levels of all 10 genes in the eIF5A gene family were downregulated (Figure 1E).

Under suitable growth conditions, *eif5a^{mut}* lines displayed distinct phenotypic alterations (Figure 1C and D). Compared with the K326, the *eif5a^{mut}* lines exhibited notably shorter main stems, concomitantly resulting in a significant decrease in leaf number. More importantly, the attenuated growth vigor of mutants compromised leaf

development quality, which may hinder subsequent economic activities related to tobacco and diminish its commercial value. These findings provide preliminary evidence that eIF5A genes, as eukaryotic translation initiation factors, modulate tobacco growth and thereby influence tobacco quality.

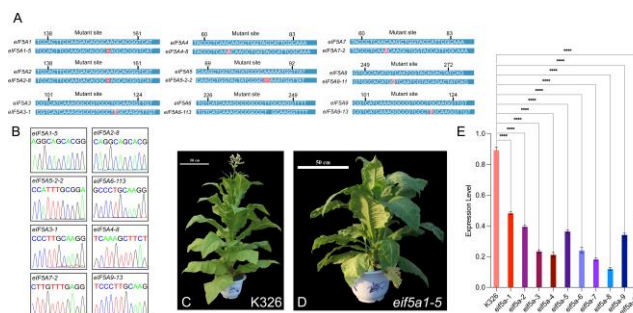


Figure 1. CRISPR knockout target sites of eIF5A family genes and mutant phenotype. (A) For the mutated regions of nucleotide sequences, each eIF5A family gene only shows one type of mutation in a segment of the sequence. The numbers indicate the base positions in the sequence, and the red marking denotes the mutation form in the background of K326. (B) Peak plot of mutated nucleotide sequence. The peak plot showing the mutation pattern of the eIF5A-8 gene was not presented since no homozygous mutants of this gene were obtained. (C) Mature *Nicotiana tabacum* cv. K326. (D) Mature *Nicotiana tabacum* cv. *eif5a1-5*. (E) RT-qPCR validation of *eif5a*.

2.2 Analysis of the eIF5A

2.2.1 Conservation Analysis of the eIF5A

By comparing the amino acid sequences of the eIF5A from 20 plant species, we obtained a conserved sequence of 134 amino acids for the eIF5A (Figure 2A). Overall, the eIF5A is highly conserved, particularly at positions 32-45 and 72-90. For non-conserved sites, their evolutionary directions are generally consistent.

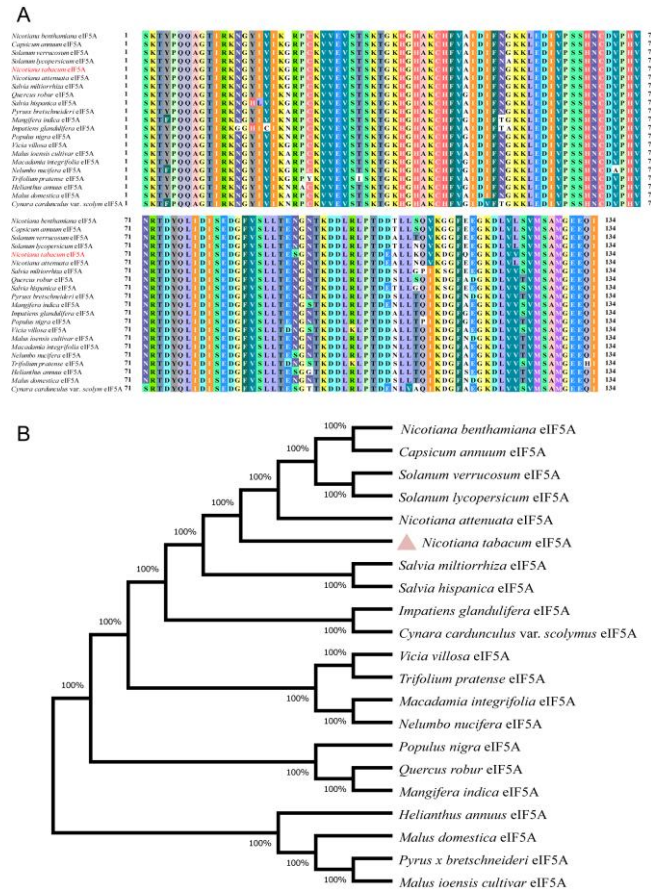


Figure 2. Conservation analysis of the eIF5A. (A) Conservation analysis of the amino acid sequence of the eIF5A gene of 20 species. (B) The Neighbor-Joining tree of the eIF5A gene on CDS of 20 species.

2.2.2 Analysis of the homologous family of the eIF5A in *N. tabacum*

We constructed a phylogenetic tree (Figure 2B) using the Neighbor-Joining method with the CDS sequences of the eIF5A from 20 plant species, including *N. tabacum*. All branches of the phylogenetic tree showed high support rates (BS=100%).

Based on the phylogenetic tree, the eIF5A genes of Solanaceae plants such as *Nicotiana tabacum*, *Nicotiana benthamiana*, *Nicotiana attenuata*, and *Capsicum annuum* are clustered together. They evolved relatively late in the evolutionary relationship and have closer genetic affinity. Meanwhile, the branches of *Nicotiana tabacum* and *Nicotiana benthamiana* within the genus *Nicotiana* are shorter, indicating that the eIF5A genes of species within the genus are highly conserved with small

evolutionary differences. Even for plants of different families and genera like *Salvia miltiorrhiza*, *Impatiens glandulifera*, and *Vicia villosa*, a regular topological structure is still formed in the phylogenetic tree, which suggests that the eIF5A gene is highly conserved among plants.

2.3 Analysis of the translation level of the eIF5A

2.3.1 Screening of DEGs

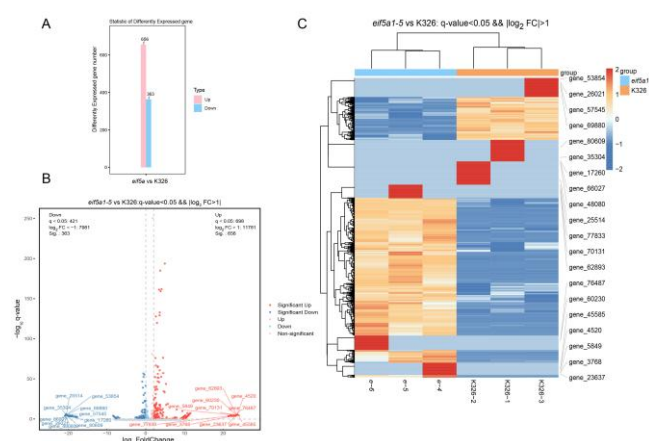
Compared to K326, we captured 1019 DEGs in the *eif5a^{mut}*, among which 656 genes showed increased expression levels, and 363 genes showed decreased expression levels (Figure 3A). By setting a p-value < 0.05 and $|\log_2FC| > 1$, we screened out the significantly differentially expressed genes and drew a heatmap. *APS1*, *TUN*, *OS6g0717800*, *Gene-62893*, *Gene-60230*, *DOF3.2*, *CRK10*, *DTX12*, *RABA5A*, *Gene-5849* were the top 10 significantly upregulated genes. *NUF2*, *CA2*, *PUB34*, *Gene-69880*, *AT1G12110*, *XBAT35*, *TAS14*, *AT3G23880*, *HRD3A*, and *Gene-57545* were the top 10 significantly downregulated genes (Figure 3B). We analyzed the expression level patterns of these genes in K326 and the *eif5a^{mut}* and drew a heatmap (Figure 3C). Different colors above the heatmap represent different groups, and three replicates (#e-1, #e-2, #e-3, #K326-1, #K326-2, #K326-3) are set for each group. The heatmap shows the top 10 significantly upregulated differentially expressed genes in K326, which are *PUB34*, *HRD3A*, *Gene_57545*, *Gene_69880*, *XBAT35*, *CA2*, *AT1G12110*, *AT3G23880*, *TAS14*, and *NUF2*. In the *eif5a^{mut}*, the top 10 significantly upregulated differentially expressed genes are *CRK10*, *DOF3.2*, *Gene_62893*, *TUN*, *Gene_60230*, *OS06g0717800*, *APS1*, *Gene_5849*, *Gene_3768*, and *RABA5A*. Moreover, the heatmap intuitively shows that there are obvious expression differences of different genes in the *eif5a^{mut}*, which is in line with our expectations.

Table 5 The symbol of genes

Gene	Symbol	Gene	Symbol
gene-4520	APS1	gene-25514	NUF2

gene-76487	TUN	gene-35304	CA2
gene-45585	OS06g0717800	gene-53854	PUB34
gene-62893	-	gene-69880	-
gene-60230	-	gene-17260	at1g12110
gene-70131	DOF3.2	gene-80609	XBAT35
gene-77833	CRK10	gene-48080	TAS14
gene-5849	-	gene-66027	at3g23880
gene-3768	DTX12	gene-26021	HRD3A
gene-23637	RABA5A	gene-57545	-
gene-45585	OS06g0717800	gene-5849	-
gene-57590	-	gene-3768	-
gene-69686	LHCB5	gene-44960	-
gene-14830	-	gene-64479	HSC-2
gene-36633	-	gene-12366	CAB36
gene-16097	CAB16	gene-44436	CAB37

295



296

297 **Figure 3.** Screening of DEGs. The symbol of the genes in Table 5. (A) Bar chart: The
298 horizontal axis represents each comparison group; the vertical axis represents the
299 number of differentially expressed genes in the comparison groups, where "Up" is the
300 number of significantly upregulated differentially expressed genes, and "Down" is the
301 number of significantly downregulated differentially expressed genes. (B) Volcano plot

for DEFs. The red and blue dots represented the significantly upregulated and downregulated genes, and the gray dots represented genes with non-significant differences. $|\log_2 FC| > 1$ and $p < 0.05$. (C) Heatmap for DEGs. The red and blue represented upregulated genes and downregulated genes.

2.3.2 Gene Ontology (GO) and KEGG pathway enrichment analysis

We presented the top 30 gene enrichment entries (Figure 4A). The vertical axis represents the gene entries, and the horizontal axis represents $-\log_{10}$ p-value. The smaller the p-value, the longer the bar, indicating a more significant enrichment of the entry. In terms of molecular function, the *eif5a^{mut}* showed more obvious differential expression in the biological function of cellular components. It exhibited extremely significant enrichment in the composition of photosystem I. In addition, there was also very significant enrichment in the composition of the chloroplast thylakoid membrane, photosystem II, and the reaction center of photosystem I. Meanwhile, there was prominent expression in aspects such as light capture in photosynthesis in biological processes and chloroplast binding in molecular functions.

Correspondingly, we also presented the top 20 pathway enrichments (Figure 4B), covering five Level 1 categories: Cellular Processes, Genetic Information Processing, Human Diseases, Metabolism, and Organismal Systems. In the figure, the horizontal axis represents the Enrichment Score. For the entries, the larger the bubble, the greater the number of differentially expressed protein-coding genes it contains. The color of the bubbles varies from blue to white to yellow and then to red. The smaller the enrichment p-value, the more significant the degree of enrichment. We were surprised to find that the pathway enrichment results showed these genes were primarily concentrated in pathways related to photosynthesis and photosynthetic proteins within metabolism, which was consistent with the results from the GO analysis.

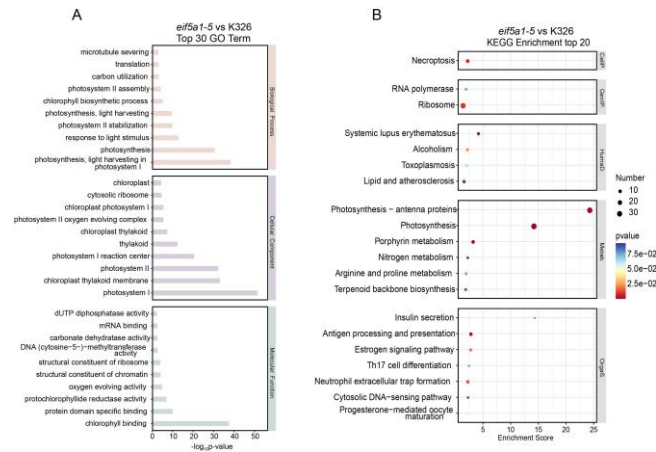


Figure 4. Perform GO and KEGG analyses on 1,019 DEGs. (A) GO analyze: The top 30 gene entries are presented. (B) KEGG analyze: The top 20 pathway enrichments are presented.

2.3.3 Quantitative PCR Analysis in translational levels and transcriptional levels.

We validated the translational levels by selecting sample(mRNA) from the 80S fraction in ribosome isolation assays of 5 differentially expressed genes by upregulated or downregulated, and the results were consistent with the above data, confirming that their translational levels were indeed upregulated or downregulated (Figure 5A and B).

We hypothesized that the *eIF5A* gene might influence the translational level in tobacco by regulating transcriptional activity. Therefore, we subsequently performed reverse-transcription quantitative PCR (RT-qPCR) assays on the top 10 upregulated and top 10 downregulated genes identified from our screening in both K326 and *elf5a^{mut}* lines. Strikingly, the results (Figure 5C and D) revealed no significant differences in the transcriptional levels of these genes between K326 and the *elf5a^{mut}*, regardless of whether they were among the top upregulated or downregulated genes. This compellingly indicates that the *eIF5A* gene solely modulates the translational level in tobacco without affecting transcriptional processes.

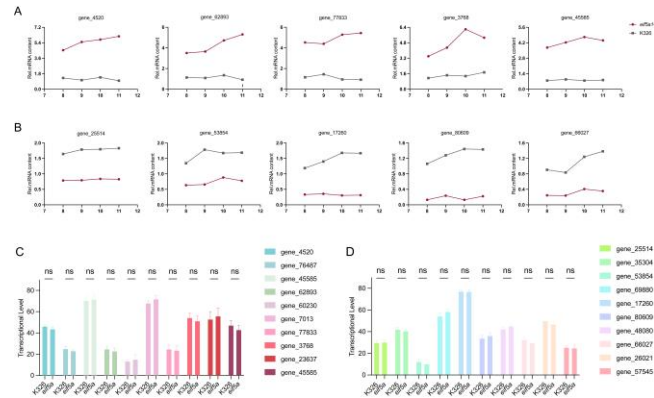


Figure 5. Verification of the translational levels and quantitative PCR Analysis in *Nicotiana tabacum*. (A) and (B) Verification of the translational levels of 5 selected genes from the top 10 upregulated or downregulated genes. The X-axis represents different fractions of ribosomes obtained from the ribosome isolation assay, and the Y-axis represents the RNA content level. (C) and (D) Reverse-transcription quantitative analysis in *Nicotiana tabacum* of the top 10 upregulated or downregulated genes.

2.4 Protein-Protein Interaction (PPI) Network of eIF5A

We used the STRING database to predict the protein interaction of eIF5A (Figure 6). The PPI network of eIF5A was predicted with a high confidence of 0.400 and visualized using Cytoscape. The interaction network consisted of 21 nodes and 129 edges, the p-value of PPF was $<10^{-16}$ and the average node degree was 12.3. Based on the degree value, the top six protein were DHS1, LOC107816705, LOC107797285, LOC107784158, LOC107783753, LOC107796463. Seventh to sixteenth were LOC107832415, LOC107832415, LOC107823125, LOC107820748, LOC107818350, LOC107801550, LOC107793525, LOC107788880, LOC107783927, LOC107780536, LOC107788104.

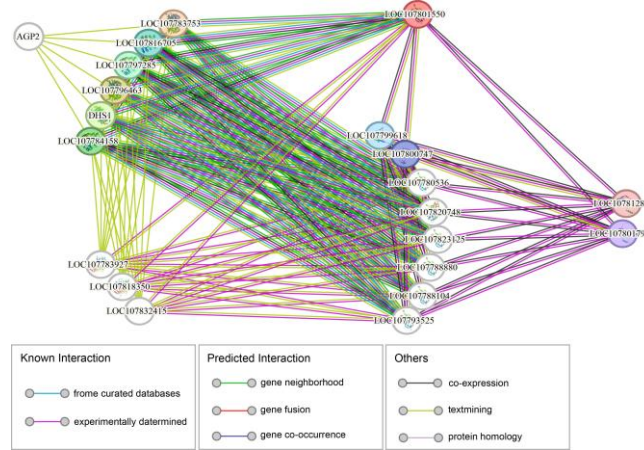


Figure 6. The protein-protein interaction network of eIF5A. Color code: query proteins and first shell of interactors. white nodes: second shell of interactors. Empty nodes: proteins of unknown 3Dstructure. Filled nodes: a 3D structure is known or predicted.

3.Discussion

Tobacco, as an important economic crop, has promoted economic and social development all over the world. Currently, research on tobacco mainly focuses on fields such as genomics(Chen et al. 2024; Wang et al. 2024), gene editing and chromosomal engineering(Sakamoto et al. 1999; Hsu et al. 2020), the interaction between plants and pathogens(Yu et al. 2019; Yang et al. 2023; Zheng et al. 2020; Tong et al. 2022), and biomedical production research(Dobrica et al. 2021; Tremblay et al. 2010).As a translation initiation factor, eIF5A and its hypusination modification are essential for understanding their roles in plant development and stress responses(Palfi et al. 2021). However, there are very few reports on the research of the eIF5A gene in tobacco at present. Meanwhile, to consolidate the important position of tobacco in economic development, it is imperative to carry out corresponding research.

Using CRISPR technology, we knocked out 10 family genes of eIF5A and obtained homozygous eIF5A mutants (Figure 1A and B). Under suitable growth conditions, the mutants showed extremely obvious phenotypic characteristics (Figure 1C and D). Compared with K326, the *EIF5A*^{mut} had shorter stems and fewer leaves. This is consistent with the phenotypes of *EIF5A*^{mut} reported in other plant species(Ren et al. 2013; Feng et

al. 2007; Xu et al. 2015). Meanwhile, due to damaged growth, the leaf quality was significantly reduced, which greatly diminished the economic value of the *eif5a^{mut}*.

Firstly, we explored the conservation of the eIF5A. We randomly downloaded the CDS sequences of the eIF5A from 19 plant species on the NCBI website, which cover different families, genera and species. By comparing the sequences of the eIF5A in 20 plant species, we found that the eIF5A is highly conserved among plants, which is confirmed by both amino acid sequence comparison (Figure 2A) and evolutionary relationship analysis (Figure 2B). This may be the result of the eIF5A being a eukaryotic translation initiation factors, and the high conservation ensures the normal execution of its functions. Certainly, eIF5A also exhibits evolutionary relationships among different plant species. Regarding the evolutionary sites of conserved sequences, their evolutionary trends tend to converge, suggesting that its primary functions may be consistent across the growth and development processes of various plants. Meanwhile, the evolutionary nature of the eIF5A implies that it may have undiscovered functions that have emerged over its long developmental history. Such it promotes the translational elongation of proline-rich sequences and plays an important role in the hydrolysis of peptidyl-tRNA during the termination process (Schuller et al. 2017). Exploring the potential biological functions of highly conserved genes is an interesting and surprising activity.

By isolating ribosomes, a total of 1019 differentially expressed genes (DEGs) were captured, among which 656 genes were upregulated, and 363 genes were downregulated (Figure 3A). *APSI*, *TUN*, *OS6g0717800*, *Gene-62893*, *Gene-60230*, *DOF3.2*, *CRK10*, *DTX12*, *RABA5A*, *Gene-5849* were the top 10 significantly upregulated genes. Most of these genes are involved in various biochemical reactions in organisms, such as protein modification, phosphorylation, and signal transduction. *NUF2*, *CA2*, *PUB34*, *Gene-69880*, *AT1G12110*, *XBAT35*, *TAS14*, *AT3G238880*, *HRD3A*, and *Gene-57545* were the top 10 significantly downregulated genes (Figure 3B). Most of them encode various proteins, such as Absciscic acid and environmental

stress-inducible protein, E3 ubiquitin-protein ligase, U-box domain-containing protein, Beta-carotene hydroxylase 2. It indicates that the eIF5A gene is crucial for the expression of upstream and downstream genes in plants(Liu et al. 2008; Feng et al. 2007). Among the top 10 downregulated genes, eIF5A may be an important component of its promoter element(Farache et al. 2022), ribosome function is impaired, or the initiation, elongation, and termination processes of protein translation are disrupted in *eif5a^{mut}* (Schuller et al. 2017; Dias et al. 2012).As for the upregulated genes, we speculate that the possible reason is that the presence of the eIF5A reduces the stability of its mRNA(Hoque et al. 2017), weakening the expression of its gene. Specifically, it may degrade mRNA through the nonsense-mediated mRNA decay (NMD) pathway (Hoque et al. 2017), or compete with other translation initiation factors (such as eIF4E) for ribosomes to hinder the assembly of initiation complexes(Hoque et al. 2017; Seoane et al. 2022).But more, As a core translation factor, mutations in eIF5A lead to global or local disruption of protein synthesis (Ribosome stalling, insufficient translation of key proteins). This "translational stress" is sensed by cells as a stress signal, which in turn activates a series of stress response pathways and ultimately upregulates the expression of defense/compensatory genes downstream of these pathways(Mandal et al. 2016).In these reports, the eIF5A gene is mostly involved in the suppression of aberrant transcripts. Whether it silences gene expressions beneficial to the organism still requires further investigation.

We particularly noticed that *Gene_57590* and *LHCB5* were significantly expressed both in K326 and the *eif5a^{mut}*, but relatively, they were still significantly upregulated in the *eif5a^{mut}* (Figure S1). Given their high expression levels and the expression differences after mutation, we have reasons to suspect that these two genes play an indispensable role in the life activities of *N. tabacum*. Through enrichment analysis of them, the results showed that the two genes both encoded antenna proteins in photosynthesis, which are Chlorophyll a-b binding protein. At the same time, some genes also had obvious expressions in the *eif5a^{mut}*, such as *Gene_44960*, *Gene_14830*,

HSC-2, and *Gene_36633*. However, their expressions were still downregulated compared with those in K326. Most of these genes were involved in the regulation of metal ion binding and were also related to the activation of the MAPK pathway. The expressions of *CAB36*, *CAB16*, and *CAB37* were extremely significantly upregulated in the *eif5a^{mut}*, and their expression levels were more than three times higher than those in K326. *CAB36* and *CAB16* are chlorophyll a-b binding proteins, and *CAB37* is an antenna protein, all of which are related to the photosynthesis of tobacco.

Through the GO analysis and KEGG analysis of the DEGs(Figure 4A and 4B), it was found that the upregulated genes were mainly enriched in aspects such as light harvesting, chlorophyll binding, and chloroplast thylakoid membrane in photosynthesis, while the downregulated genes were mainly enriched in aspects such as protein folding, unfolded protein binding, and nuclear RNA-directed RNA polymerase complex, which are related to protein modification and the regulation of the central dogma (Figure S2). We speculate that in *N. tabacum*, besides serving as a eukaryotic translation initiation factor and participating in the transcription and translation of DNA, eIF5A may also plays a significant role in the process of photosynthesis. Current reports do not directly indicate that eIF5A can directly participate in the photosynthetic process in *N. tabacum*, but Liu(Liu et al. 2019) found that PhDHS plays important roles in chloroplast development, photosynthesis, and leaf senescence in petunia. Instead, it is involved in regulating the translation and modification of proteins related to photosynthesis, thereby influencing the progression of photosynthesis (Leschevin et al. 2024; Belda-Palazon et al. 2016; Liu et al. 2019).

To address our suspicion that eIF5A might regulate translation levels by influencing gene transcription, we analyzed the transcriptional levels of the top 10 upregulated and top 10 downregulated genes screened. As expected, neither the top 10 upregulated nor downregulated genes showed significant differences in transcriptional levels between K326 and the mutant, emphasizing that eIF5A does not affect gene transcription but directly regulates translation (Figure 5C and D). This phenomenon has

472 been reported in other species previously, but it is the first time it has been observed in
473 tobacco(Barba-Aliaga et al. 2024; Schuller et al. 2017). Thus, we confirmed that in *N.*
474 *tabacum*, eIF5A, as a eukaryotic translation initiation factor, regulates the expression
475 of genes such as *APSI*, *TUN*, *Gene_57590*, and *LHCB5* at the translational level
476 without affecting their transcription, modulating pathways related to photosynthesis to
477 influence tobacco growth and quality.

478 Finally, we predicted the interacting proteins of eIF5A (Figure 6). Among the top
479 six identified proteins, all are associated with deoxyhypusine synthesis. DHS1
480 functions as a deoxyhypusine synthase(Njuguna et al. 2006), and Wang(2005) found
481 that the eIF5A gene family is upregulated concomitantly with enhanced DHS mRNA
482 levels during tomato fruit senescence and softening. This suggests that a single DHS
483 gene and its homologous proteins in tomato are involved in activating four distinct
484 eIF5A isoforms, demonstrating the functional interplay between DHS and eIF5A in
485 plant growth and development. LOC107816705, LOC107797285, LOC107784158,
486 and LOC107796463 encode deoxyhypusine hydroxylases, while LOC107783753
487 represents a deoxyhypusine synthase-like isoform X1. Thus, we hypothesize that eIF5A
488 may regulate deoxyhypusine synthesis through protein-protein interactions.
489 Intriguingly, eIF5A also exhibits potential interactions with ribosomal 40S and 60S
490 components (e.g., LOC107799618 and LOC107812846), indicating that as a regulator
491 of eukaryotic translation initiation, it may directly engage with ribosomes to modulate
492 downstream gene expression. Meanwhile, additional uncharacterized signaling
493 pathways involved in eIF5A regulation warrant further exploration.

495 **Conflict of interest statement**

496 The authors declare that there is no conflict of interest.

498 **Author Contributions Statement**

499 L.Q W and Y W, along with Y.B Q, completed most of the basic experiments and
500 drafted the initial manuscript. L.Q W, Y W, X.X H, and Y W obtained the key data.

S.B Z, Y P, and X.Y Z participated in the experiments. Z Mand J.P G designed the overall experimental approach of the article, provided guidance throughout the experiments, and contributed to the writing and revision of the paper.

Data Availability Statement

All the data and plant material are available from the corresponding author.

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